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Antrocamphin A, an Anti-inflammatory Principal from the Fruiting Body of *Taiwanofungus camphoratus*, and Its Mechanisms

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The fungus *Taiwanofungus camphoratus* is commonly used for medicinal purposes in Taiwan. It is used as a detoxicant for food poisoning and considered to be a precious folk medicine for hepatoprotection and anti-inflammation. In this study, a lipopolysaccaride (LPS)-challenged ICR mouse acute inflammation model and a LPS-induced macrophage model were used to evaluate the anti-inflammatory activity of *T. camphoratus*. Ethanol extract of *T. camphoratus* significantly inhibited expression of iNOS and COX-2 in the liver of LPS-challenged acute inflammatory mice. The ethyl acetate fraction and its isolated compound, antrocamphin A, significantly suppressed nitrite/nitrate concentration in LPS-challenged RAW 264.7 cells. Antrocamphin A showed potent anti-inflammatory activity by suppressing pro-inflammatory molecule release via the down-regulation of iNOS and COX-2 expression through the NF-κB pathway. This study, therefore, first demonstrates the bioactive compound of *T. camphoratus* and illustrates the mechanism by which it confers its anti-inflammatory activity.

KEYWORDS: *Taiwanofungus camphoratus*; anti-inflammation; antrocamphin A; NO; PGE2; NF-κB

INTRODUCTION

Inflammation is a central feature of many pathological conditions (*1*). Prevention and treatment of inflammation are important applications for natural products (*2*). *Taiwanofungus camphoratus* (also known as niu-chang-chih) (*3*) is a precious fungus that is used in Taiwan as a folk medicine for treating liver cancer, food and drug intoxications, diarrhea, abdominal pains, and hypertension (*4*). Due to their perceived efficacies, the wild fruiting bodies of *T. camphoratus* are sold for around U.S. $15000 per kilogram (*5*). Over the past few years, several studies have investigated the anti-inflammatory activities of *T. camphoratus* (*6*−*14*); however, most studies focused on crude extracts of mycelia or fruiting body. Papers that discuss the activity of a single compound isolated from *T. camphoratus* are rare.

Superoxide anion is an inflammatory mediator produced by neutrophils. Chen and colleagues demonstrated that antrocamphin A, antcin A, and antcin B isolated from *T. camphoratus* possessed inhibition against *N*-formaylmethionyl-leucyl-phenylanine (fMLP) induced superoxide anion production (*11*). Recently, a group of succinic and maleic derivatives have been reported to possess anti-inflammatory activity. However, the mechanism of these active compounds has not yet been investigated (*4*). In the present study, a lipopolysaccaride (LPS)-challenged ICR mouse acute inflammation model was used to study the anti-inflammatory activity of *T. camphoratus*. Following a bioactivity-guided fractionation procedure, a potent anti-inflammatory compound, antrocamphin A, was identified from the fruiting body of *T. camphoratus*. To elucidate the anti-inflammatory mechanism of antrocamphin A, the effects of antrocamphin A on activation of the NF-κB pathway, as well as the inflammatory response of the downstream mediators iNOS and COX-2, were studied.

MATERIALS AND METHODS

***Taiwanofungus camphoratus* and Chemicals.** Wild fruiting bodies of *T. camphoratus* were collected from Liugui, Kaohsiung County, Taiwan, in July 2007 and identified by Prof. S.-Y. Wang (National Chung Hsing University, Taiwan). A voucher specimen (WSY 07-001) was deposited in the Core Laboratory of Plant Metabolomics at Chung-Hsing

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University. Dulbecco's modified essential medium (DMEM) and other cell culture reagents including fetal bovine serum (FBS) were obtained from Gibco BRL (Invitrogen, Grand Island, NY). Dimethyl sulfoxide (DMSO), penicillin, tryspin−EDTA, Tris-HCl, sodium dodecyl sulfate (SDS), lipopolysaccharide (LPS, *Escherichia coli* 0127:138), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents used in this study were of reagent or HPLC grade.

Animals. Male ICR mice (4 weeks old, 25−28 g) were purchased from BioLasco (Taipei, Taiwan) and housed at a temperature of 25 ± 2 °C, with 55 ± 5% relative humidity, a 12 h light/dark cycle (6:00 a.m.−6:00 p.m. light), and food and water ad libitum. Mice were allowed to adapt to their environment for at least one week before testing and were transferred to the laboratory at least 1 h before the start of experiments. All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (*15*) and Taiwan laws relating to the protection of animals and were approved by the local ethics committee.

Extract Preparation, Isolation, and Compound Identification. Five hundred and eighty grams of air-dried fruiting bodies of *T. camphoratus* was extracted exhaustively with 95% ethanol (EtOH) at ambient temperature. Total crude EtOH extract (TCE) was concentrated under vacuum to yield a residue (183.9 g) and then partitioned between ethyl acetate (EtOAc) and H_2O to give an EtOAc-soluble fraction (TCE-EA) and an H_2O-soluble fraction (TCE-W). The EtOAc-soluble fraction was further separated by chromatography over silica gel (60−80 mesh), eluted with an *n*-hexane/EtOAc gradient (*n*-hex/EtOAc, 95:5; 90:10; 85:15; 80:20; 70:30; 60:40; 50:50; 40:60, 0:100, each 1 L) to produce 17 fractions. As fraction 1 revealed the strongest nitric oxide (NO) inhibitory activity, it was further separated by high-performance liquid chromatography (HPLC) using a Luna silica column (250 × 10 mm; Phenomenex, Torrance, CA) eluted with an *n*-hex/EtOAc solvent system (*n*-hex/EtOAc = 85:15, flow rate = 3 mL/min) to obtain antrocamphin A (**1**) (retention time = 17 min). The structure of antrocamphin A (*11*) was elucidated and confirmed by spectroscopic analysis. UV spectra were recorded on a Jasco V-550 and IR spectra on a Bio-Rad FTS-40 spectrometer. Electrospray ionization−mass spectrometric spectrometry (ESIMS) and high-resolution electron-impact mass spectrometry (HREIMS) data were collected with a Finnigan MAT-95S mass spectrometer, and NMR spectra were recorded with Bruker Avance 500 and 300 MHz FT-NMR spectrometers, at 500 MHz (^{1}H) and 75 MHz (^{13}C). *d*-Chloroform ($CDCl_3$) was used for NMR analysis.

In Vivo Anti-inflammation Experiments. Mice were divided into six groups consisting of six mice each. TCE and curcumin were dissolved in DMSO and administered to mice by intraperitoneal injection with or without various concentrations of TCE (100, 300, 500 mg/kg) or curcumin (100 mg/kg) 4 h before LPS induction by intraperitoneal injection (5 μg/kg) (*16*, *17*). Control mice received vehicle (DMSO) only. Mice were sacrificed by decapitation during anesthesia with ethyl ether 12 h after LPS injection. Blood was collected by eye bleeding or cardiac puncture in EDTA tubes and centrifuged at 500*g* for 10 min at 4 °C, and NO concentrations in serum were measured indirectly using the Griess reaction (*18*). Whole cell, cytosolic, and nuclear proteins were collected from liver 12 h after LPS injection. The livers were quickly removed, flash frozen in liquid nitrogen, and milled. Whole proteins were isolated using Mammalian Protein Extraction Reagent (Cayman Chemicals, Ann Arbor, MI), and whole cell extracts were prepared as previously described (*19*). The cytosolic and nuclear extracts were prepared according to protocol 78833 using a Nuclear and Cytoplasmic Extraction Reagents (NE-PER) kit (Pierce Biotechnology, Rockford, IL). The protein content was quantified according to the Bradford method with absorbance at 595 nm (*20*).

Nitric Oxide Inhibitory Assay. Effects of TCE and antrocamphin A on NO production were measured indirectly by analysis of nitrite levels using the Greiss reaction (*21*, *22*). Briefly, RAW 264.7 cells grown in a 75 cm^2 culture dish were seeded in 96-well plates at a density of 2 × 10^5 cells/well. Cells were cultured at 37 °C in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator as recommended by the American Type Culture Collection (ATCC). Adherent cells were then incubated with or without 1 μg/mL of LPS for 24 h, in the absence or presence of compounds. Nitrite concentration (as an estimate of NO production) was measured using the supernatant from the RAW 264.7 cells by the Griess reaction (*18*).

Determination of Prostaglandin E_2 Production. RAW 264.7 cells (2 × 10^5 cells/well) seeded on a 96-well plate were pretreated with 500 μM aspirin for 3 h to inactivate endogenous COX-1, washed twice with PBS, and then treated with antrocamphin A (1, 5, 10, 20 μg/mL) and curcumin (10 μg/mL) for 1 h. The cells were then incubated for 16 h in fresh DMEM medium with or without 1 μg/mL of LPS. The concentration of PGE_2 produced from endogenous arachidonic acid was measured in cell culture supernatants by an ELISA kit according to the supplier's instructions (Cayman Chemicals).

Preparation of Whole Cell, Cytosolic, and Nuclear Extracts. The preparation of whole cell extract was as previously described (*19*). Briefly, RAW 264.7 cells (5 × 10^6 cells/well) seeded on 6 cm dishes were treated with different concentrations of antrocamphin A (1, 5, 10, 20 μg/mL), stimulated with LPS (1 μg/mL), and incubated at 37 °C, 5% CO_2 for 16 h before COX-2 and iNOS assay. For the measurement of NF-κB (p50 and p65), IκBα, and IKK, cells were treated for 1 h. Curcumin (10 μg/mL) was used as a reference compound. For whole cell extracts, cells were lysed in Mammalian Protein Extraction Reagent (Cayman Chemicals), lysates were centrifuged at 4 °C for 10 min, and the supernatant was collected. The cytosolic and nuclear extracts were prepared as above using a kit (Pierce Biotechnology). The protein content was quantified by absorbance at 595 nm according to the Bradford method (*20*).

RNA Isolation and RT-PCR Analysis. Total RNA was isolated from live mouse tissue using Trizol reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Quantitative real-time reverse-transcription Polymerase Chain Reaction (RT-PCR) analysis for iNOS, COX-2, and GADPH mRNA were performed using Applied Biosystems (AB) detection instruments and software. The AB system incorporates a gradient thermocycler and a 96-channel optical unit. For the quantitative analysis of mRNA expression, the AB system was employed using DNA binding dye, SYBR Green, for the detection of PCR products. The melting point, optimal conditions, and specificity of the reaction were first determined using a standard procedure. The working stock solution of SYBR Green was 1:100 (Bio-Rad). Quantitative PCR was carried out in a 48-well plate with 10 pmol of forward and reverse primers and the working solution SYBR Green, using a PCR master mix, under the following conditions: 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 30 s. G3PDH, a housekeeping gene, was chosen as an internal standard to control for variability in amplification because of differences in starting mRNA concentrations. The sequences of the PCR primers were as follows: iNOS, forward 5′-TCC TAC ACC ACA CCA AAC-3′, reverse 5′-CTC CAA TCT CTG CCT ATC C-3′; COX-2, forward 5′-CCT CTG CGA TGC TCT TCC-3′, reverse 5′-TCA CAC TTA TAC TGG TCA AAT CC-3′; G3PDH, forward 5′-TCA ACG GCA CAG TCA AGG-3′, reverse 5′-ACT CCA CGA CAT ACT CAG C-3′. The copy number of each transcript was calculated as the relative copy number normalized by GAPDH copy number.

Western Blot Analysis. RAW 264.7 cells were incubated with or without various concentrations of antrocamphin A, curcumin, and 1 μg/mL of LPS to measure the expression of iNOS, COX-2, IKK, IκBα, and NF-κB proteins. Whole cell, cytoplasmic, and nuclear protein extracts were collected from treated and untreated cells. Equal amounts of protein were loaded in 5−7% gradient gels and resolved by SDS-PAGE at 300 mA for 90 min. The size-separated proteins were electroblotted onto poly-(vinylidene difluoride) (PVDF) membrane (Immobilon, Millipore, Bedford, MA) at 100 V for 1 h. The membranes were incubated in blocking buffer (10% w/v skim milk in TBST buffer) for 1 h and then incubated with anti-iNOS (1:1000) or anti-COX-2 (1:1000) (Cayman Chemicals), anti-NF-κB (1:500) (Abcam, Cambridge, U.K.), anti-IKK (1:1000) and anti-IκBα (1:1000) polyclonal antibody (Cell Signaling Technology, Danvers, MA), and anti-actin (1:500) monoclonal antibody (Sigma). After two washes with 0.1% TBST (TBS containing 0.1% Tween 20), the membranes were incubated with anti-rabbit secondary antibodies conjugated with horseradish peroxidase and detected by the enhanced chemiluminescence reagents (ECL, Pierce). β-Actin levels were measured as a loading control.

Statistical Analysis. Data are expressed as means ± SE. The significance of differences between group means was analyzed by analysis of variance (ANOVA) using Dunnett's test. Differences were declared to be significant if P values were < 0.05 (indicated by *; ** indicates $P < 0.01$).

Table 1. Effects of Ethanol Extract of *T. camphoratus* on NO Production in LPS-Challenged Mouse Blood Serum[a]

group/dose (mg/kg)	NO (μM)
control	37.79 ± 1.87
TCE 100	22.6 ± 0.44***
TCE 300	15.04 ± 0.3***
TCE 500	8.79 ± 0.11***
curcumin 100	10.79 ± 0.27***

[a] The crude extract (TCE, 100−500 mg/kg) of *T. camphoratus* was injected 4 h before inflammation induction. Control represents the response in animals treated with LPS (5 μg/kg) only. $n = 6$ animals. The data are shown as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, indicate significant differences.

RESULTS AND DISCUSSION

Effect of TCE on NO Production in LPS-Induced Mouse Blood Serum. To investigate the anti-inflammatory activity of *T. camphoratus*, we first immersed the fruiting body in 95% ethanol to get the EtOH crude extract (TCE). NO produced by iNOS and prostaglandins, the biosynthesis of which originates from the enzyme COX-2, have been implicated as important mediators in the process of inflammation (*23*). We determined the level of inflammation by detecting the NO concentration in serum of 4-week-old mice challenged by intraperitoneal injection of LPS (5 μg/kg). The mice preinjected with various concentrations of TCE showed NO inhibition activity in a dose-dependent manner (**Table 1**). Dosages of 100 and 300 mg/kg showed 58 and 87% inhibition, respectively, compared with the control. Furthermore, mice pretreated with 500 mg/kg of TCE completely inhibited NO production with an efficiency similar to that of mice pretreated with a well-known anti-inflammatory compound, curcumin, at 100 mg/kg.

Effects of TCE on iNOS and COX-2 Protein Expression in LPS-Induced Mouse Liver Tissue. To further confirm the causes of NO reduction, we detected the protein expression level of iNOS and COX-2 in liver (**Figure 1**). LPS-challenged mice injected with TCE at various concentrations showed only a very low level of iNOS protein, indicating that the reduction of NO concentration in serum results from the iNOS inhibition. The expression of COX-2, another inflammatory response-related protein, was also suppressed in a dose-dependent manner; in particular, mice treated with 500 mg/kg crude extract showed a significant effect that was similar to that of the curcumin-treated group.

Isolation of Anti-inflammation Compound from *T. camphoratus*. To identify compounds with high potential of anti-inflammatory response, we further divided the TCE into two fractions: an ethyl acetate fraction (TCE-EA) and an aqueous fraction (TCE-W). We found that in LPS-stimulated RAW 264.7 cells the most effective TCE-EA suppressed NO production at 48% in comparison with the control. These results suggested that TCE-EA possesses NO inhibitory activity. To identify the active compound with NO inhibitory activity, TCE-EA was further separated into 17 fractions (TCE-EA-1−17) by chromatography. The derivative 17 subfractions were continuously evaluated for their NO inhibitory activity using the LPS-induced NO production cell model at the concentration of 50 μg/mL. TCE-EA-1 revealed the strongest NO inhibition activity with 87% inhibition (see Figure S1 in the Supporting Information) and thus chosen as target to identify the constituents. TCE-EA-1 was further purified by using HPLC to obtain a yellow oil-like compound, **1**. The molecular formula of compound **1** was determined to be $C_{15}H_{18}O_3$ by ESI-MS ($[M + H]^+$, m/z 247.14) and HRESI-MS ($[M + H]^+$ ion, m/z 247.1335). The UV spectrum showed absorption maxima at 265 nm, and the IR spectrum showed characteristic absorption

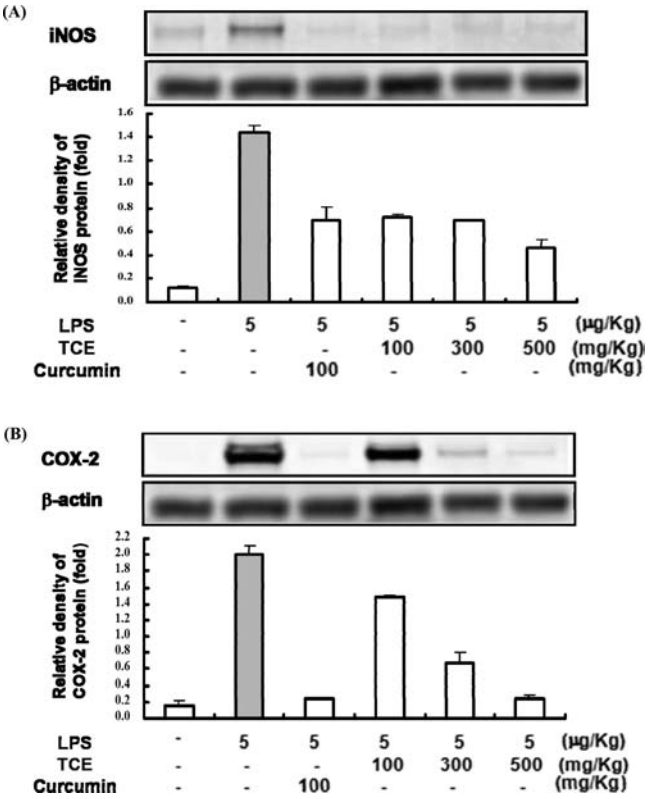

Figure 1. Effects of the ethanol extract of *T. camphoratus* (TCE) on iNOS (**A**) and COX-2 (**B**) protein expression in liver of LPS-challenged mice. Mice were administered by intraperitoneal injection with or without various concentrations of TCE or curcumin (100 mg/kg) 4 h before LPS (5 μg/kg) injection. Data represent one of six similar results.

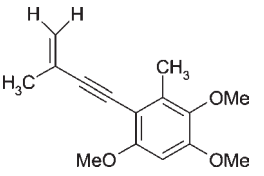

Figure 2. Chemical structure of antrocamphin A.

Table 2. Effects of Antrocamphin A on NO, PGE_2 Production, and Cytotocixity in LPS-Challenged RAW 264.7 Cells[a]

compound/dose (μg/mL)	NO (μM)	PGE_2 (pg/mL)	cell viability (%)
control	17.43 ± 0.42	429.04 ± 28.47	100 ± 6.45
antrocamphin A 1	18 ± 0.36	411.5 ± 20.92	93 ± 4.13
antrocamphin A 5	14.68 ± 0.54**	360.9 ± 23.95	92 ± 5.49
antrocamphin A 10	11.14 ± 0.18***	237.96 ± 83.85**	89 ± 3.22
antrocamphin A 20	7.27 ± 0.18***	24.62 ± 15.36***	79 ± 3.54
quercetin 10	16.96 ± 0.2	6.82 ± 5.2***	76 ± 4.6

[a] The ethanol fraction isolated compound, antrocamphin A, was administrated 1 h before inflammation induction. Control represents the response in RAW 264.7 cells treated with LPS (1 μg/mL) only. $n = 3$. The data are shown as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, indicate significant differences.

bands due to the presence of a C≡C stretch at 2198 cm^{-1} and a C=C stretch in an aromatic ring at 1594, 1492, and 1462 cm^{-1}. In the ^1H NMR spectra, **1** showed three methoxy groups (δ 3.862, 3H, s; δ 3.860, 3H, s; δ 3.69 3H, s), an aromatic methyl group (δ 2.39, 3H, s), an aromatic proton (δ 6.31, 1H, s), and a 3-methylbut-3-enynyl group (δ 5.35, 1H, br s; δ 5.23, 1H, br s; δ 2.0, 3H, s). MS, UV, IR, and ^1H NMR analyses indicated that the spectral data of **1** were in complete agreement with those of

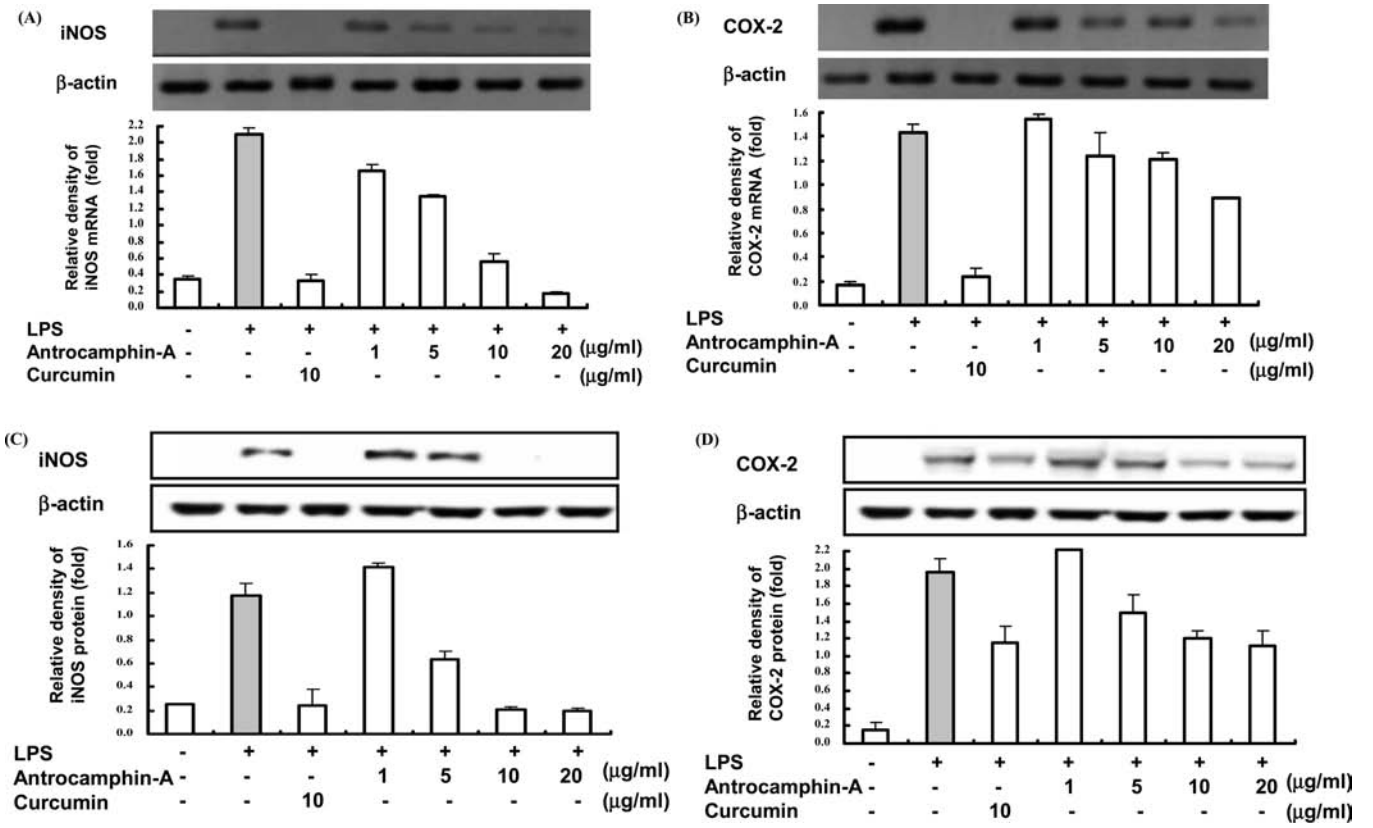

Figure 3. Effects of antrocamphin A on iNOS and COX-2 mRNA (**A**, **B**) and protein (**C**, **D**) expression in LPS-challenged macrophages. RAW 264.7 cells were pretreated for 1 h with various concentrations of antrocamphin A or curcumin (10 μg/mL) and then challenged by LPS (1 μg/mL). The total RNA and whole cell lysate were extracted as described under Materials and Methods.

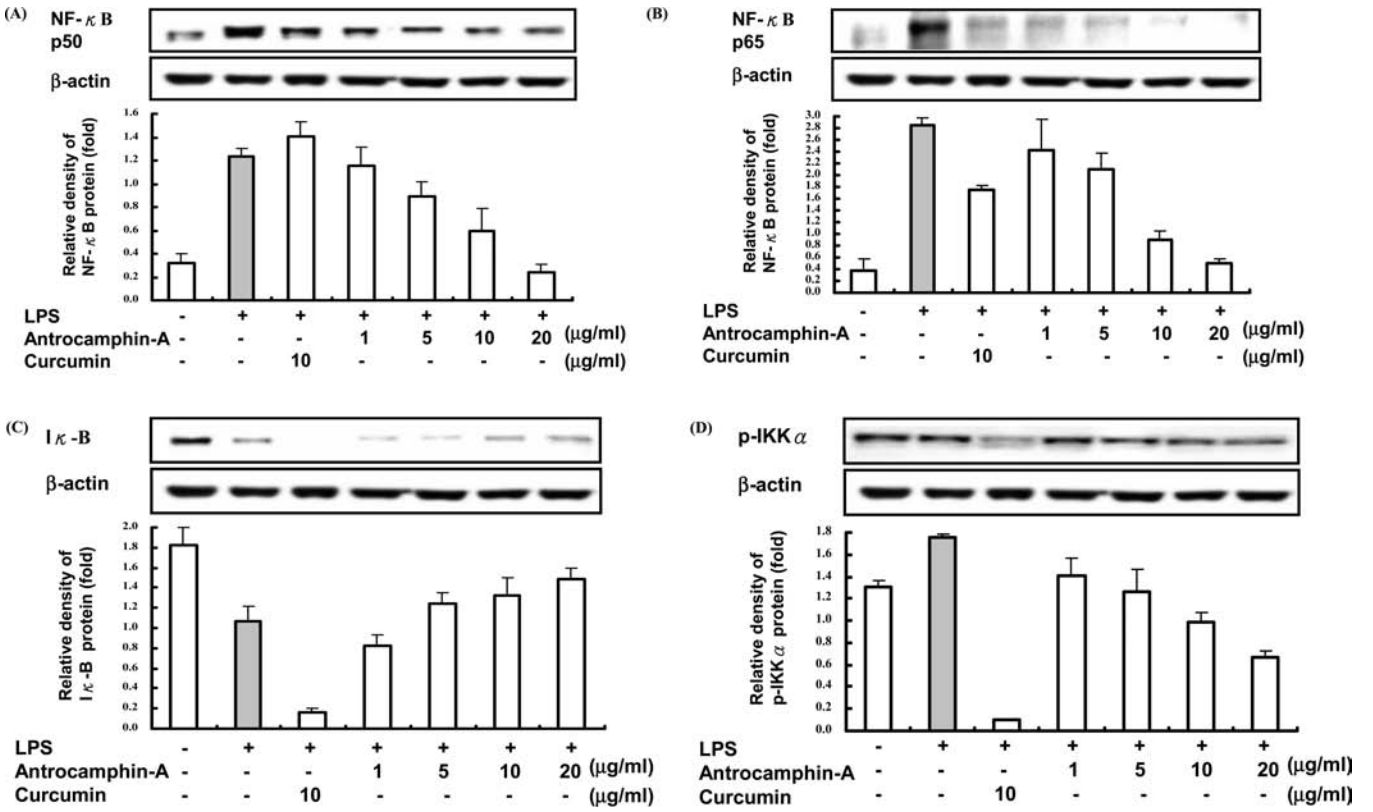

Figure 4. Effects of antrocamphin A on NF-κB p50 (**A**) NF-κB p65 (**B**), I-κB (**C**), and IKK (**D**) expression in LPS-challenged macrophages. RAW 264.7 cells were pretreated for 1 h with various concentrations of antrocamphin A or curcumin (10 μg/mL) and then challenged by LPS (1 μg/mL). The nuclear NF-κB (p50 and p65) and the cytosolic I-κB (**C**) and IKK (**D**) were extracted as described under Materials and Methods.

antrocamphin A (**Figure 2**) (*11*). The total yield of antrocamphin A in the ethanolic extract of *T. camphoratus* was 0.378% (w/w). Chen et al. first published the anti-inflammatory activity of antrocamphin A and zhankulic acids on fMLP-induced superoxide generation model (*11*). Because of the polarity difference, we isolated zhankulic acid compounds in higher polarity fractions such as TCE-EA-7 and TCE-EA-10. According to our screening data, the anti-inflammation activity of zhankulic acids was lower than that of antrocamphin A in the same NO inhibition assay.

Elucidation of Anti-inflammation activity of Antrocamphin A. We evaluated the effect of antrocamphin A on anti-inflammatory activity by detecting the production of pro-inflammatory molecules including NO and PGE_2 (**Table 2**) in LPS-challenged macrophages. Both NO and PGE_2 productions were suppressed in a dose-dependent manner. Antrocamphin A inhibited NO production from 18 ± 0.36 to 7.27 ± 0.18 μM and inhibited PGE_2 generation from 411.5 ± 20.92 to 24.62 ± 15.36 pg/mL. More importantly, antrocamphin A worked efficiently at 20 μg/mL, reducing the concentration of NO and PGE_2 back to normal compared with control without cytotoxicity. The mRNA and protein expressions of antrocamphin A treated macrophages showed that iNOS and COX-2 are down-regulated at both the transcriptional and translational levels (**Figure 3**). That suppressive efficiency is dose-dependent confirmed our previous finding of NO and PGE_2 inhibition. iNOS protein was completely suppressed at a dosage of 10 μg/mL (**Figure 3C**), and expression of COX-2 protein was also suppressed in a dose-dependent manner under different dosages of antrocamphin A treatment (**Figure 3D**). We hypothesize that the inhibition of iNOS and COX-2 expressions by antrocamphin A could be the result of poor nuclear translocation of NF-κB. The translocation of NF-κB to the nucleus has been shown to be required for NF-κB-dependent transcription following LPS stimulation (*24*). As shown in **Figure 4**, the nuclear fractions of NF-κB subunits p50 and p65 were enhanced in the presence of LPS (1 μg/mL) alone compared with unchallenged microphages. RAW 264.7 cells cotreated with antrocamphin A decreased NF-κB accumulation in the nucleus in a dose-dependent manner. IκB is a NF-κB inhibitor that can be degraded through ubiquitination after phosphorylation by IKK (*25*). In this study, we found that the expression of IκB increased with antrocamphin A treatment (**Figure 4C**) in parallel with an IKK decrease (**Figure 4D**). On the basis of these results, we assume that the poor nuclear translocation of NF-κB could be due to NF-κB inactivation resulting from stabilization of IκB. Furthermore, IKK phosphorylation was involved in this down-regulation of the anti-inflammatory response.

In conclusion, we confirm that the potent anti-inflammatory compound, antrocamphin A, inhibits the production of pro-inflammatory molecules such as NO and PGE_2 in LPS-stimulated macrophages. We also demonstrate that this anti-inflammatory effect occurs by the down-regulation of iNOS and COX-2 expression at both the transcriptional and translational levels via suppression of transcription factor NF-κB. The present study supports the traditional use of *T. camphoratus* for anti-inflammation treatment. Moreover, the results obtained in this study may provide a valuable reference for the future development of pharmaceutical or functional food applications of *T. camphoratus*.

Supporting Information Available: Figure S1 (effects of 17 subfractions of the ethyl acetate extract of *T. camphoratus* on NO production in LPS-induced macrophages). This material is available free of charge via the Internet at http://pubs.acs.org.

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